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PRINCIPAL INVESTIGATOR: Erica McCoy

CONTRACTING ORGANIZATION: University of Colorado Health Sciences Center
Aurora, CO 80045

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Erica McCoy

Email: erica.mccoy@uchsc.edu

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14. ABSTRACT

Homeobox transcription factor Six1 has been identified as a critical mediator of tumorigenesis and metastasis in a number of organ systems and has been implicated in epithelial to mesenchymal transitions (EMT) during normal development. Our research is aimed at utilizing mouse models to understand its role in the onset and progression of breast cancer. Most significantly, we have determined that Six1 is indeed sufficient to induce tumor formation in the mammary glands of mice genetically engineered to inducibly overexpress the gene. The latency for tumor formation is between 12-15 months and the tumors that arise in these animals are very aggressive and have morphological features of EMT, a phenomenon that has recently been suggested to contribute to metastasis. Further molecular analysis of these tumors will allow us to more carefully dissect the role of Six1 in tumor onset. Additionally, in a number of animals that do not develop tumors, a hyperplastic phenotype is observed. Future experiments will involve turning off Six1 expression after tumor formation to determine if Six1 is required for tumor maintenance, thus determining the potential benefits of targeting Six1 in a therapeutic setting.

15. SUBJECT TERMS
Six1, PyMT, p53DN, epithelial to mesenchymal transitions (EMT), tumorigenesis, mammary gland

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INTRODUCTION:

Homeobox transcription factor Six1 is a critical mediator of embryonic development, where it stimulates proliferation and survival of progenitor populations as well as epithelial to mesenchymal transitions (EMT) (1,2). Interestingly, Six1 expression is often found to be reinitiated in tumors where evidence suggests it stimulates proliferation, survival, and EMT in tumor cells to cause tumor progression and metastatic disease. Six1 overexpression is documented in a number of tumor types, including ovarian cancer, hepatocellular carcinoma, Wilms' tumor, rhabdomyosarcomas and breast cancer (2-7). Our research is aimed at utilizing mouse models to understand its role in the onset and progression of breast cancer. We are currently using an inducible mouse model to overexpress Six1 in differentiated cells of the mammary gland to determine if Six1 is sufficient for inducing tumor formation. Additionally, we are using a retroviral transplant model to overexpress Six1 in mammary progenitor cells to see if the gene is able to drive tumor formation differently if present in a less differentiated cell type. Future experiments will involve turning off Six1 expression in both models after tumor formation to determine if Six1 is required for tumor maintenance, thus determining the potential benefits of targeting Six1 in a therapeutic setting. Our final aim in these studies is to identify if cyclinA1 is the Six1 transcriptional target responsible for mediating Six1's tumorigenic potential.

BODY:

Aim 1: Determine the role of Six1 overexpression in mammary gland tumorigenesis. (Months 1-24)

(A) Evaluate the consequence of chronic Six1 overexpression in mammary gland epithelial cells using a tetracycline inducible mouse model (months 1-18).

Our progress for this aim has been substantial. Long term induction of Six1 expression (TOSIX bitransgenic animals overexpress the gene upon treatment with doxycycline) results in mammary tumor formation in a subset of animals after approximately 12-15 months. Although we have not analyzed the majority of animals dedicated to this study, 3/15 analyzed thus far have presented with mammary tumors. These tumors have been characterized by our collaborating pathologist, who determined that they are high grade adenocarcinomas with features of EMT, including sarcomatoid regions and loss of e-cadherin expression (a marker of epithelial cells). (figures 1 and 2).

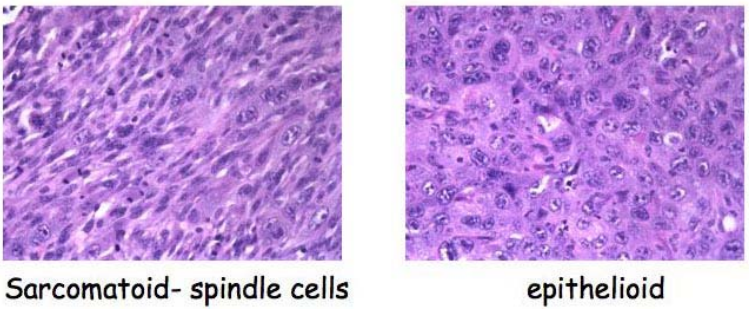

Fig. 1. Six1 induces an EMT in tumors in vivo. Two histological sections (40x) from a single tumor arising in a TOSIX1 mouse induced with Dox. The tumor has regions that are both sarcomatoid (containing many spindle cells) and epithelioid, demonstrating that regions of the tumor are undergoing EMT in vivo. Animal sacrificed 18 months after Dox induction.

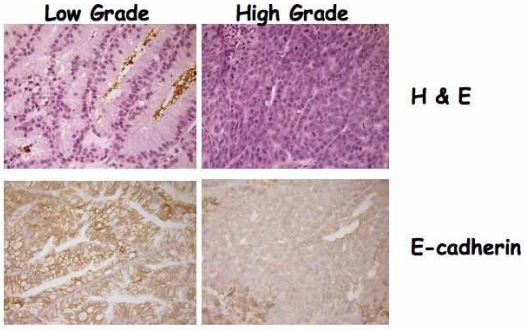

Fig. 2. Analysis of TOSIX tumor provides evidence of EMT. Low and High grade regions can be observed in tumors, with low grade regions exhibing robust E-cadherin expression and high grade regions having marked downregulation of E-cadherin.

In addition to the tumor phenotype, we observe a hyperplastic phenotype in the mammary glands of animals induced to overexpress Six1 (figure 3). These differences were compared by quantifying the epithelial content of TOSix and MTB control animals induced with doxycycline.

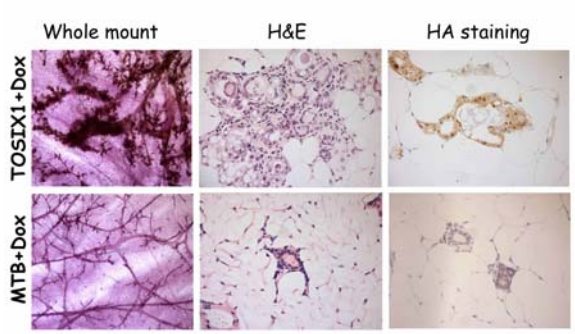

Fig. 3. Six1 overexpression leads to hyperplasia, and abnormal alveologenesis. TOSIX+Dox upper panels, MTB+Dox lower panels. Whole mounts, H&Es, and HA staining to identify transgene expression are shown. TOSix1 gland shown from 4922 line, although the same phenotype is observed in the 6239 line.

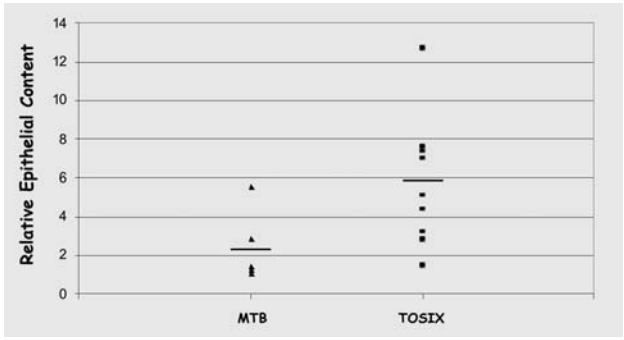

Fig. 4. Epithelial content is increased in TOSIX mice induced with Dox as compared to MTB mice induced with Dox. H& E sections from either MTB+ Dox or TOSIX+ Dox mice (from both the 4922 and 6239 lines) were scanned using an Aperio T3 Scanscope at an effective resolution of 0.46 microns/pixel square. Image analysis was performed using ImageJ version 1.37 running on Java version 1.5. Mammary epithelial tissue is isolated based on specified color values from white field background. Resulting data is a percent composition value illustrating the relationship between mammary epithelial tissue and non-epithelial tissue.

(B) Determine the effect of retroviral-mediated Six1 overexpression in mammary gland progenitor cells (months 1-18).

The results from long-term Six1 expression as discussed above suggest that the latency for Six1-mediated tumorigenesis will be longer than a year. Unfortunately, this study has only been in progress for approximately 9 months and the animals expressing Six1 via transplant have not yet developed tumors. However, these animals will be maintained until at least 18 months to determine if similar tumor latency will be observed in this model as the transgenic model described above. We have identified two oncogenenic mutations with which we want to test the effect of combined Six1 expression. For these studies, we have harvested primary mammary epithelial cells from wildtype animals and then retrovirally transduced them with either Six1 and the polyoma middle T antigen (PyMT) or dominant negative form of p53 with a R175H mutation commonly associated with human breast cancer (p53DN) (controls including Six1 alone or the oncogenic mutation alone are included). These cells are then transplanted into the cleared fat pad of three week old recipient wildtype mice. Progenitor cells within the population will give rise to a mammary ductal network that will express Six1 along with either PyMT or p53DN.

PyMT is known to give rise to tumors with a very short latency in this model (approximately 3 months following transplant) (8). Therefore we will monitor animals to determine if Six1 is able to accelerate tumorigenesis or affect metastasis. At this time, we have preliminarily observed that Six1 does not affect primary tumor latency or growth in this model. However, we are currently pursuing the identification of any differences in metastasis (including differences in metastatic lesion size, growth rate, and organ site of occurance).

Overexpression of p53DN in a transgenic mouse model failed to exhibit a tumor phenotype, but did render the mammary gland more susceptible to tumor induction with chemical carcinogens and other oncogenes (9). Therefore, we do not expect p53DN overexpression in our retroviral model to lead to tumor formation on its own. Rather, we hypothesize that the addition of Six1 overexpression will lead to tumor formation in these experiments. At this time, we have setup a number of animals on this experiment and are monitoring them for tumor development.

(C) Determine if mammary gland tumorigenesis (if observed) or additional phenotypes are dependent upon Six1 for maintenance using the inducible and retroviral overexpression models outlined in aims 1a and 1b (months 18-24).
At this time, we are currently waiting for a significant number of animals to present with tumors to attempt withdrawing Six1 expression in both models.

Aim 2: Examine the dependency of Six1 on cyclin A1 for mammary gland proliferation and tumorigenesis. (Months 18-36)
As the tumor latency with Six1 overexpression is long, we have not begun the experiments associated with this aim where we will cross TOSIX1 mice with Cyclin A1 knockout mice to determine whether cyclin A1 is required for Six1-mediated tumorigenesis. Once we have further analyzed the results from Aim 1, we will continue to pursue Aim 2.

KEY RESEARCH ACCOMPLISHMENTS:

- Identified that long term induction of Six1 expression in an inducible mouse model leads to hyperplasia as well as aggressive tumor formation.
- Noted that Six1 does not affect tumor latency or growth when combined with the PyMT oncogene.
- Setup studies to identify the role of Six1 in PyMT-mediated metastasis and p53DN-mediated tumorigenesis.

REPORTABLE OUTCOMES:

Research presented in the form of a poster at the International Association of Breast Cancer Research (IABCR) meeting in Montreal, Canada, September 2006. Additionally, research will be presented in poster form at the Gordon Conference for Mammary Gland Biology in Newport, RI this June. See appendix for poster abstracts.

Grants that resulted from this work include a grant from the American Cancer Society entitled "The Role of Six1 in EMT and Tumor Progression". ($150,000 per year direct costs/ for 4 years. The grant runs from 5/1/07-4/30/11.

CONCLUSIONS:

The results from studies completed thus far suggest that Six1 is indeed capable of initiating mammary tumorigenesis. The tumors that arise in animals induced to express Six1 are very aggressive adenocarcinomas displaying high-grade regions with features of EMT. As EMT

has been implicated in metastasis, these results suggest that Six1 is an oncogene not only capable of initiating mammary tumor onset, but may be capable of initiating the first stages of aggressive tumor progression. These results are quite compelling, given the clinical data that Six1 is overexpressed in 50% of primary breast cancers and 90% of metastatic lesions (1,2). Our ultimate goal is to identify Six1 as a legitimate therapeutic target. As Six1 expression is normally only critical for embryonic development, lost in the adult, and re-expressed in cancers, targeting Six1 in a clinical setting may successfully treat cancer while avoiding damage to normal adult tissues, thus limiting side-effects. An important experiment we will be conducting in the future involves reversing Six1 expression in the tumors that arise in our Six1 overexpressing models. If these tumors regress, it will be a significant finding that may lead to the development of therapies designed to target Six1.

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APPENDICIES:

Poster abstract submitted for IABCR conference in September 2006:

In Vivo Role of the Six1 Homeoprotein in Mammary Gland Tumorigenesis

E.M. McCoy1, N. Abbey2, R.D. Coletta2, D. Micalizzi1, P. Jedlicka3,L. Chodosh4, H.L. Ford1,2, 1Program in Molecular Biology, 2Department of Obstetrics and Gynecology, 3 Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado, USA 4Department of Cancer Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

Human Six1 is a homeodomain-containing transcription factor that is critical for cell proliferation, survival, and epithelial to mesenchymal transition (EMT) during normal development. In addition to its developmental role, overexpression of Six1 has been detected in a number of human cancers, including breast cancer, where it is linked to both proliferation and metastasis. As many as 50% of primary breast cancers and 90% of metastatic lesions overexpress the gene, in part due to gene amplification. Six1 can transform a mammary epithelial cell line, but no work has been done to show the effects of Six1 overexpression *in vivo.* We have established an inducible, mammary-specific Six1 overexpression model by crossing MMTV-rtTA mice to TetO-Six1 mice, and are using this model to test whether Six1 overexpression leads to mammary tumors, as well as to dissect the molecular mechanism by which Six1 influences tumorigenesis *in vivo*. In multiparous mice induced to overexpress Six1 in the mammary gland, a hyperproliferative phenotype is observed. Furthermore, preliminary data indicate that invasive mammary adenocarcinoma showing features of EMT is observed with long latency and constitutive Six1 overexpression in the nulliparous state. Data from this model suggests that inappropriate expression of Six1 promotes tumorigenesis and that this transformation occurs through an EMT-like process. This inducible model provides us with a system to examine whether removal of Six1 expression can reverse the phenotypes, thereby addressing whether Six1 is a viable drug target. Importantly, Six1 is not necessary for most normal adult tissues, and thus therapies directed against Six1 may not lead to the severe side effects seen with more conventional treatments.

Poster submitted for Gordon Conference on Mammary Gland Biology scheduled for June, 2007:

In Vivo Role of the Six1 Homeoprotein in Mammary Gland Tumorigenesis

E.M. McCoy1, N. Abbey2, P. Jedlicka3,L. Chodosh4, H.L. Ford1,2, 1Program in Molecular Biology, 2Department of Obstetrics and Gynecology, 3 Department of Pathology, University of Colorado Health Sciences Center, Denver, Colorado, USA 4Department of Cancer Biology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania, USA

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